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Dissimilar Plasmids Isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) Contain Identical *opd* Genes

LINDA L. HARPER, C. STEVEN McDANIEL, CHARLES E. MILLER, AND JAMES R. WILD*

Department of Biochemistry and Biophysics, Texas A&M University System, College Station, Texas 77843

Received 28 March 1988/Accepted 28 June 1988

The *opd* (organophosphate-degrading) gene derived from a 43-kilobase-pair plasmid (pSM55) of a *Flavobacterium* sp. (ATCC 27551) has a sequence identical to that of the plasmid-borne gene of *Pseudomonas diminuta*. Hybridization studies with DNA fragments obtained by restriction endonuclease digestion of plasmid DNAs demonstrated that the identical *opd* sequences were encoded on dissimilar plasmids from the two sources.

Pseudomonas diminuta MG and a *Flavobacterium* sp. (ATCC 27551) have the ability to degrade a broad spectrum of organophosphorous triesters (2, 4) by virtue of a constitutively expressed organophosphorous acid anhydrase (11, 14). This enzyme stereospecifically hydrolyzes the triester bond common to organophosphorous pesticide molecules without a phosphorylated enzyme intermediate (7). Neither of the strains appears to utilize organophosphorous molecules as nutrients, nor do the substrates appear to be directly toxic to the bacterial cultures. These independent soil isolates encode organophosphorus detoxification genes (*opd*) on large plasmids (10, 13), and the plasmid-borne genes have been cloned (8, 10, 12; C. S. McDaniel, Ph.D. dissertation, Texas A&M University, College Station, 1985). The *Pseudomonas* gene has been sequenced (8).

Recently, it has been shown that crude whole-cell extracts from both *P. diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) were able to degrade diisopropyl fluorophosphate (1), an analog of the nerve gas Soman. Purified enzyme preparations of the cloned *Pseudomonas* gene product have been shown to specifically degrade diisopropyl fluorophosphate (D. Dumas, J. Wild, and F. Raushel, submitted for publication). Furthermore, methyl parathion-degrading *Pseudomonas* isolates that possess DNA homologous to the *opd* gene have been obtained (3). The studies reported in this paper describe the sequencing of the *Flavobacterium* plasmid-encoded gene and the comparison of that genetic system with the *opd* gene and the plasmid of the *Pseudomonas* strain.

Escherichia coli required a host promoter immediately 5′ of the *opd* sequence. For that reason, the subcloning of the 7.3-kb *opd*-containing fragment from the *Flavobacterium* sequence (pWWM44) was achieved by *Pst*I digestion and insertion of the fragment into M13mp11 and by screening for whole-cell organophosphorous acid anhydrase activity (9) under *lac* control. Those clones possessing lactose-inducible organophosphorous acid anhydrase activity (as well as clones having reverse orientation, i.e., inactive isolates) were sequenced. Similarities in the restriction endonuclease digestion patterns between the two *opd* genetic regions prompted the sequencing of the *Pst*I fragment from the *Flavobacterium* plasmid by using the techniques and DNA primers previously described for the *Pseudomonas* gene (8). The two genes were shown to be identical, with the possible exception of a G-to-C transversion at base 295 (Fig. 1). This identity extended beyond the putative gene sequence in both the 5′ (62-base-pair [bp]) and 3′ (286-bp) directions and included the entire *Pst*I fragment. By using the Align subroutine of the SEQ program (Intelligenetics, Palo Alto, Calif.), the 5′ region common to both genes was compared with the *Pseudomonas* prototype promoters and the *nif* promoter (5) (see sequences below). The 5′-flanking region of the *opd* open reading frame contains the invariant dinucleotides with a critical spacing of 10 bp, which corresponds to the recognition sequence (GG-10 bp-GC) for the core of sigma-60 rather than the consensus sequences of *E. coli* (TTGACA-16 or 17 bp-TATAAT) recognized for the core of sigma-70 (5).

Pseudomonas	NT-GGCNNNNNNNNTGCT
opd	CTCGGCACCAGTCGCTGCAAGCAGAGTCGTAAGCAATCGCAAGGGGGCAGCATG
nif	CT-GGCA-CNNNNNTTGCA

The initial cloning of the *Flavobacterium* sp. *opd* gene region was performed as described previously (10) by using partial *Eco*RI digestions of the 43-kilobase-pair (kb) plasmid from the *Flavobacterium* sp.; however, no enzymatic activity was observed for the subclones containing the large fragment. Earlier work on cloning the *Pseudomonas opd* gene had demonstrated that its heterologous expression in

In addition to a potential *Pseudomonas*-like promoter sequence in the 5′-flanking region of the *opd* gene, there is also a potential ribosome-binding site (AAGGGGG) just 5′ of the presumed translational start codon (underlined above).

The nonidentical nature of the two separately isolated plasmids was demonstrated by using a pair of *Pst*I fragments from the *P. diminuta* plasmid (pCMS1) as probes against the plasmid DNA of the *Flavobacterium* sp. (Fig. 2). The cloning of the two *Pst*I fragments (C″ and D) from pCMS1 which were used in this study has been described previously (8). The C″ fragment from the *Pseudomonas* plasmid (1,326

* Corresponding author.

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1-59     5'-CT GCA GCC TGA CTC GGC ACC AGT CGC TGC AAG CAG AGT CGT AAG CAA TCG CAA GGG GGC
60-119     AGC ATG CAA ACG AGA AGG GTT GTG CTC AAG TCT GCG GCC GCG AGA ACT CTG CTC GGC GGC
120-179    CTG GCT GGG TGC GCG ACG TGG CTG GAT CGA TCG GCA CAG GCG ATG CGA TCA ATA CGT GCG
180-239    CGT CCT ATC ACA ATC TCT GAA GCG GGT TTC ACA CTG ACT CAC GAG GAC ATC TCG GCA GCT
240-299    CGG CAG GAT TCT TGC GTG CTT GGC CAG AGT TCT TCG GTA GCG CAA AGC TCT AGC GCA AAA
300-359    GGC TGT GAG AGG ATT GCG CGC CAG AGC GGC TGG CGT GCG AAC GAT TGT CGA TGT GTC GAC
360-419    TTT CGA TAT CGG TCG CGA CGT CAG TTT ATT GGC CGA GGT TTC GCG GGC TGC CGA CGT TCA
420-479    TAT CTG GCG GCG ACC GGC TTG TGG TTC GAC CCG CCA CTT TCG ATG CGA TTG AGG TAT GTA
480-539    GAG GAA CTC ACA CTA GTT CTT CCT GCG GTG AGA TTC AAT ATG GCA TCG AAG TAC ACC GGA
540-599    ATT AGG GCG GGC ATT ATC AAG GTC GCG ACC ACA GGC AAG GCG ACC CCC TTT CAG GAG TTA
600-659    GTG TTA AAG GCG GCC GCC CGG GCC AGC TTG GCC ACC GGT GTT CCG GTA ACC ACT CAC ACG
660-719    GCA GCA AGT CAG CGC GAT GGT GAG CGA GGC AGG CCG CCA TTT TTG AGT CCG AAG CTT GAG
720-779    CCC TCA CGG GTT TGT ATT GGT CAC AGC GAT GAT ACT GAC GAT TTG AGC TAT CTC ACC GCC
780-839    CTG CTG CGC GGA TAC CTC ATC GGT CTA GAC CAC ATC CCG CAC AGT GCG ATT GGT CTA GAA
840-899    GAT AAT GCG AGT GCA TCA CCG CTC CTG GGC ATC CGT TCG TGG CAA ACA CGG GCT CTC TTG
900-959    ATC AAG GCG CTC ATC GAC CAA GGC TAC ATG AAA CAA ATC CTC GTT TCG AAT GAC TGG CTG
960-1019   TTC GGG TTT TCG AGC TAT GTC ACC AAC ATC ATG GAC GTG ATG GAT CGC GTG AAC CCC GAC
1020-1079  GGG ATG GCC TTC ATT CAC TGA GAG TGA TCC CAT TCT ACG AGA GAA GGG CGT CCC ACA GGA
1080-1139  AAC GCT GGC AGG CAT CAC TGT GAC TAA CCC GGC GCG GTT CTG TGT CAC CGA CTT GCC GTG
1140-1199  CAT GAC GCC ATC TGG ATC CTT CCA CGC AGC GGC CAC TAT TCC CCG TCA AGA TAC CGA ACG
1200-1259  ATG AAG TCG CGC ATC GAT CGA TAG GCA TCT TCA ATG TGA TCA GGG CTG CCA CCT CCA AAG
1260-1319  CCG GTG GCC ACC CCT GTC GAT AGT CTT GAG GGA CGG TAG CGA CGA CCG TGC TTT TCG TGA
1320-1326  ACT GCA G-3'
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FIG. 1. Nucleotide sequence of *Flavobacterium opd* gene fragment. Base 295, which may represent a G-to-C transversion with respect to the *Pseudomonas* gene, is underlined. Bold type indicates the putative coding sequence for the gene. Base numbers are given on the left.

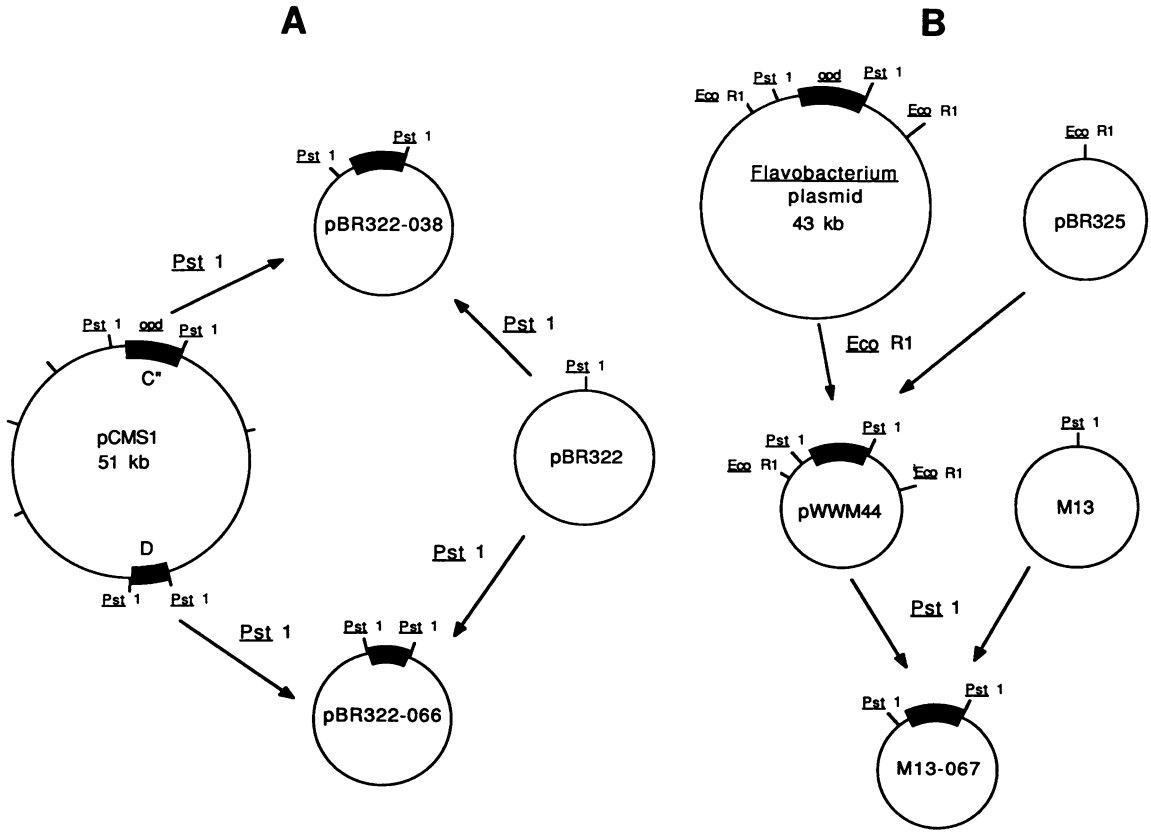

FIG. 2. Derivation of plasmids used in this study. (A) *P. diminuta* plasmid pCMS1 (51 kb) is the native plasmid containing the *opd* gene. Two pBR322 clones containing either the *opd* gene (1.3 kb, C'' fragment) or a fragment from the *Pseudomonas* plasmid lacking the *opd* gene (0.9 kb, D fragment) were constructed. (B) *Flavobacterium* sp. plasmid (43 kb) is the native plasmid containing the *opd* gene. pWWM44 is the pBR325 derivative containing an *Eco*RI fragment of the native plasmid which possessed the *opd* gene sequence. M13-067 is the M13mp11 derivative of pWWM44 which contains the 1.3-kb active fragment *opd*.

bp) containing the *opd* gene was shown by sequence analysis to be identical to the same-size fragment from the *Flavobacterium* plasmid. A second *Pst*I fragment (D) of approximately 900 bp was chosen as a probe since it was separated from the region containing the known homology by approximately 22 kb, as estimated by a preliminary restriction digest map of the *Pseudomonas* plasmid (McDaniel, Ph.D. dissertation). For all of the hybridization studies, the methods of Southern (15) were used.

Figure 3 demonstrates the strong hybridization of both *Pseudomonas* and *Flavobacterium* plasmid DNAs with the 1,326-bp (C′) fragment containing the *opd* gene sequence. The *Pst*I-digested plasmids differed considerably in their restriction profiles (Fig. 3A). There appeared to be a single plasmid in the *Flavobacterium* strain, although it was present in several forms. Upon restriction, a single hybridizing band was observed for each of the two plasmid sources of the gene (Fig. 3B), and the overall restriction endonuclease pattern is similar to that observed for the isolated plasmid (unpublished observations).

When the 900-bp fragment (D) was used as a probe against both plasmid DNAs (Fig. 4A and B), it hybridized to DNA in the control (*Pst*I-digested pCMS1) and the unrestricted *Pseudomonas* plasmid. However, the fragment failed to hybridize to either the native or the restricted plasmid DNA from *Flavobacterium* sp. These results are consistent with the restriction site data reported previously (10) and reiterate the dissimilarity of the two plasmids.

Other examples of genetic homology among *Pseudomonas* degradative plasmids, such as TOL, NAH, and SAL, indicate evolutionary interrelationships. Appreciable homology has been found between the chlorobenzoate degradative plasmid pAC25 and plasmids such as TOL, SAL, and a *Pseudomonas* antibiotic resistance plasmid, even when

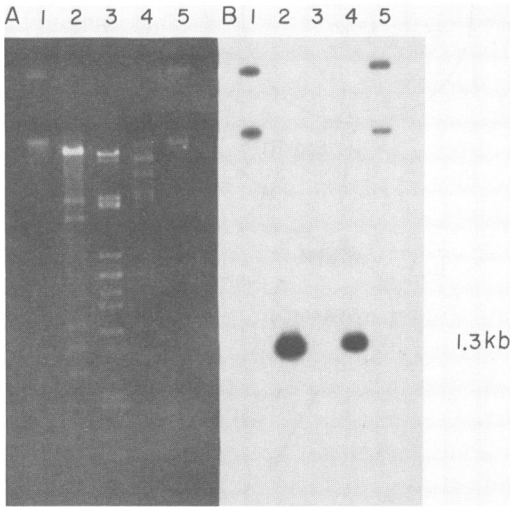

FIG. 3. Southern blotting and hybridization of the 1.3-kb probe with plasmid DNAs from *P. diminuta* and a *Flavobacterium* sp. (A) Agarose gel electrophoresis of the *opd*-encoding plasmid DNAs. Lanes: 1 and 2, CsCl-purified pCMS1 DNA from *P. diminuta* unrestricted and restricted with *Pst*I, respectively; 3, *Pst*I-restricted lambda DNA as a molecular-size marker; 4 and 5, CsCl-purified plasmid DNA from a *Flavobacterium* sp. restricted and unrestricted with *Pst*I, respectively. (B) Hybridization transfer membrane (GeneScreen Plus, Dupont, NEN Research Products) blot of the gel in panel A to which ^{32}P-labeled purified fragment DNA was hybridized. The lanes correspond to those described for panel A.

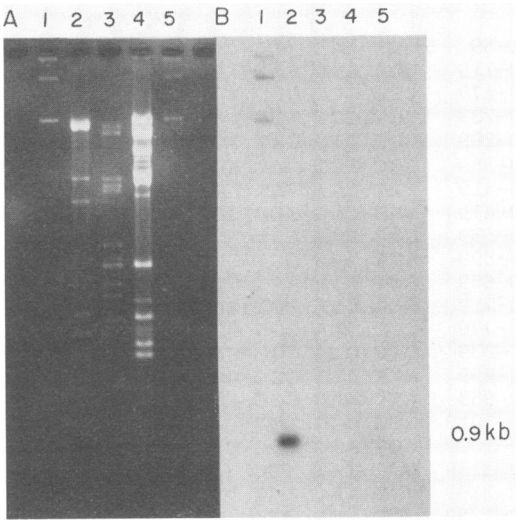

FIG. 4. Southern blotting and hybridization of the 0.9-kb probe with plasmid DNAs from *P. diminuta* and a *Flavobacterium* sp. (A) Agarose gel electrophoresis of the *opd*-encoding plasmid DNAs. Lanes: 1 and 2, CsCl-purified pCMS1 DNA from *P. diminuta* unrestricted and restricted with *Pst*I, respectively; 3, *Pst*I-restricted lambda DNA as a molecular-size marker; 4 and 5, CsCl-purified plasmid DNA from a *Flavobacterium* sp. restricted and unrestricted with *Pst*I, respectively. (B) Hybridization transfer membrane (GeneScreen Plus, Dupont) blot of the gel in panel A, to which ^{32}P-labeled purified fragment DNA was hybridized. The lanes correspond to those described for panel A.

these plasmids differ in incompatibility and host range characteristics (6). This finding suggests that there may be more-extensive homology between the two parathion-degradative plasmids than just the *opd* gene itself. Studies are under way to determine the nature of the junction between the homologous and nonhomologous regions of the two plasmids containing the *opd* genes. Sequence analysis in the flanking regions may provide insight into a mechanism of genetic transfer for a degradative plasmid-borne gene in soil microorganisms.

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